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Prolactin Antagonist, hPRL-G129R, In Several Breast Cancer Cell
Lines

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13. ABSTRACT (Maximum 200 Words)

We compared the differential gene expression profile of 4 human breast cancer cell lines following treatment with hPRL and its antagonist. Among the genes identified of an apoptosis array, the bcl-2 gene was of particular interest, and we found that this gene was upregulated in 3 of the 4 cell lines that were treated with PRL. To further confirm these results, real-time PCR analysi was used to detect bcl-2 mRNA, in 11 human breast cancer cell lines after treatment with hPRL or its antagonist. Our data suggest that Bcl-2 is upregulated in response to hPRL stimulation & is competively inhibited by the antagonist in the majority of the cell lines tested. We propose that the anti-apoptotic role of hPRL in breast cancer is mediated, at least in part, through regulation of bcl-2.

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Introduction

To gain insight into the molecular basis of human prolactin (hPRL) antagonist induced apoptosis, we compared the differential gene expression profile of four human breast cancer cell lines following treatment with hPRL and its antagonist (hPRL-G129R). Among the genes identified, the *bcl-2* gene was of particular interest. We found that *bcl-2* mRNA was up regulated in three of the four cell lines that were treated with hPRL. To further confirm these results, real time RT-PCR and ELISA analyses were used to detect *bcl-2* mRNA and Bcl-2 protein, respectively, in eleven different breast cancer cell lines after hPRL or hPRL-G129R treatment. Our data suggest that Bcl-2 is up regulated in response to hPRL stimulation and is competitively inhibited by hPRL-G129R in the majority of the cell lines tested. We propose that the anti-apoptotic role of hPRL in breast cancer is mediated, at least in part, through regulation of Bcl-2.

Body: Research accomplishments associated with each task outlined in the approved Statement of Work.

Specific Aim I: To identify genes specific to human prolactin antagonist-induced apoptosis in human breast cancer cell lines.

In this study, we examined the profile of apoptosis related genes expressed by four human breast cancer cell lines upon treatment with either hPRL or hPRL-G129R, using a newly described technique called suppression subtractive hybridization (SSH) in combination with cDNA microarrays. It was found that *bcl-2* gene expression was increased following treatment of breast cancer cells with hPRL in both estrogen receptor (ER) positive cell lines and one of two ER negative cell lines tested.

We compared all relevant apoptosis related genes expressed in human breast cancer cells. Treatment of T-47D and MCF-7 cells with hPRL up-regulated *bcl-2* expression. In contrast, T-47D cells treated with hPRL-G129R exhibited a strong up-regulation of caspases, such as caspase 3, 4, 7, 9 and 10. The Bcl-2 binding protein, BNIP$_3$, was expressed in all but the MDA-MB-468 after hPRL-G129R treatment. In the T-47D cells, the genes related to death receptors such as Serine-Threonine Kinase 1, DAXX, Tumor Necrosis Factor-Related Apoptosis Inducing Ligand and Death Domain Receptor 3 were up-regulated after hPRL-G129R treatment, although the gene for caspase 8, which is normally associated with death receptors, was not. In MCF-7 cells the gene *BAD*, an important member of the Bcl-2 family of proteins, was differentially expressed after hPRL-G129R treatment. There was no evidence of caspase expression in MCF-7 cells after hPRL-G129R treatment.

Specific Aim II: Use of a quantitative method of RT-PCR (reverse transcription "real time" PCR) to determine the mRNA copy number of the apoptotis-related gene we identified as most critical, *bcl-2*, in eleven breast cancer cell lines.

To confirm the evidence linking hPRL and Bcl-2, a quantitative method of RT-PCR was used to measure *bcl-2* mRNA expression levels, in eleven human breast cancer cell lines after treatment with hPRL or hPRL-G129R. The data from these studies suggest that hPRL acts as an apoptosis inhibitor by increasing the expression of Bcl-2 in

human breast cancer, and that hPRL-G129R competitively inhibits Bcl-2 induction by hPRL.

Figure 1: Real time quantitative measurement of *bcl-2* mRNA levels in eleven breast cancer cell lines in response to 48-h treatments with hPRL (500 ng/ml). Levels are represented as the relative change from the untreated controls (=0), and numbers are presented as mean ± SE. All samples were normalized to equivalent levels of β-actin mRNA.

FIGURE 1

CELL LINES: 1. MCF-7; 2. MDA-MB-134; 3.T-47D; 4. BT549; 5. MDA-MB-436; 6. MDA-MB-468; 7. MDA-MB-157; 8. MDA-MB-453; 9. BT474; 10. MDA-MB-231; 11. BT483.
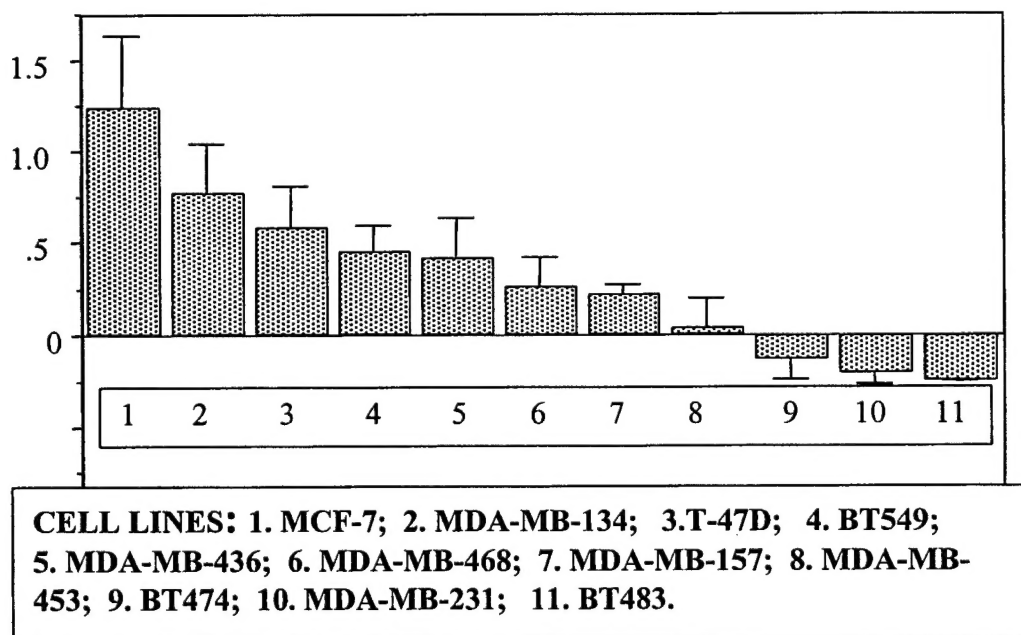
Figure 2: Real time quantitative measurement of *bcl-2* mRNA levels in eleven breast cancer cell lines in response to 48-h treatments with hPRL-G129R (500 ng/ml). Levels are represented as the relative change from the untreated controls (=0), and numbers are presented as mean ± SE. All samples were normalized to equivalent levels of β-actin mRNA.

FIGURE 2

CELL LINES: 1. MCF-7; 2. MDA-MB-134; 3.T-47D; 4. BT549;
5. MDA-MB-436; 6. MDA-MB-468; 7. MDA-MB-157; 8. MDA-MB-
453; 9. BT474; 10. MDA-MB-231; 11. BT483.
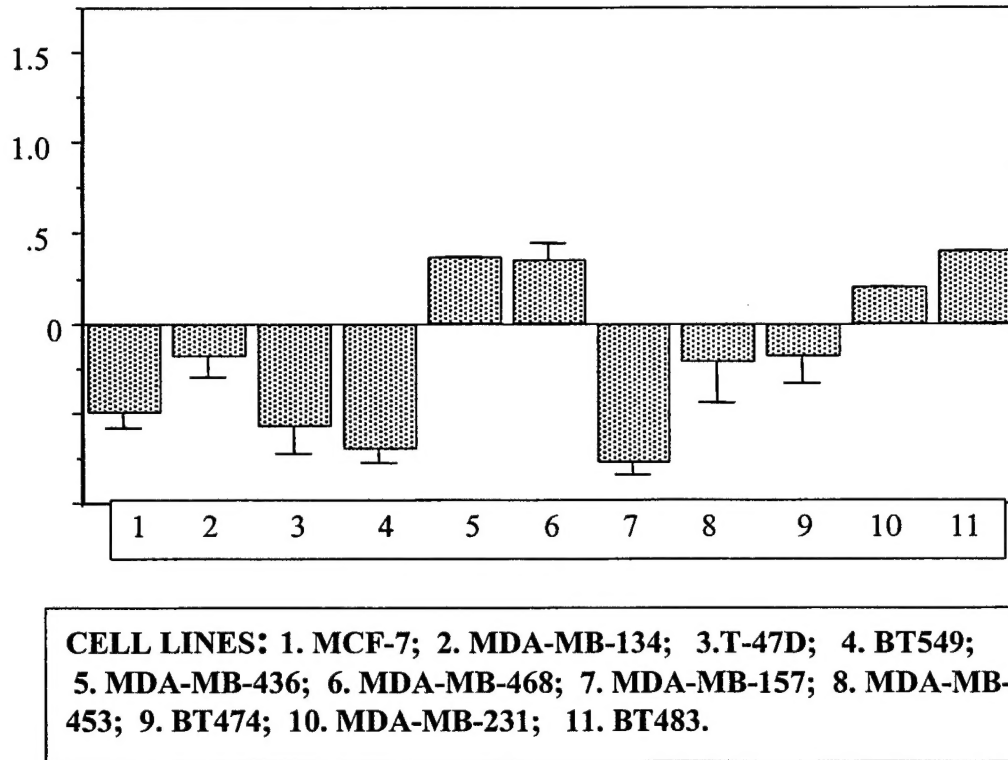
Table 1 summarizes the *bcl-2* levels from multiple quantitative real time RT-PCR runs relative to normalized levels of β-actin. The data are presented as levels of *bcl-2* in all eleven cell lines treated with hPRL, hPRL-G129R or a combination of hPRL and hPRL-G129R, and were compared to levels of *bcl-2* in the untreated controls.

In MCF-7 and MDA-MB-134 cells, hPRL treatment resulted in a highly significant ($P<0.01$) up-regulation of *bcl-2* message, while in BT-549 and T-47D cells the percent change was significant ($P<0.05$). In the remaining seven cell lines, *bcl-2* message levels were not significantly different from the untreated controls. Treatment with the antagonist resulted in significantly ($P<0.05$) decreased expression of *bcl-2* message in four of the cell lines (MCF-7, T-47D, BT-549 and MDA-MB-157) and no significant change in the other cell lines. A modest increase in *bcl-2* message expression was observed in four cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-231, BT-483) following hPRL-G129R treatment. In seven cell lines a combination treatment of hPRL-G129R and hPRL resulted in lower levels of *bcl-2* expression than the hPRL treatment

alone (MCF-7, T-47D, MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483). The combination treatment significantly ($P<0.05$) reduced *bcl-2* expression levels in MCF-7 and T-47D cell lines, whereas MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483 cells show an insignificant decrease in the level of Bcl-2.

Table 1 Fold difference of *bcl-2* message of treatments over untreated cells.

Cell line	PRL[a]	G129R[b]	PRL+G129R[c]
MCF-7	2.25 ± 0.19	0.50 ± 0.08	0.45 ± 0.15
MDA-MB-134	1.78 ± 0.27	0.82 ± 0.12	0.76 ± 0.17
T47-D	1.59 ± 0.29	0.43 ± 0.15	0.24 ± 0.03
BT549	1.46 ± 0.14	0.30 ± 0.07	1.41 ± 0.38
MDA-MB-436	1.42 ± 0.28	1.38	2.77
MDA-MB-468	1.27 ± 0.15	1.26 ± 0.09	1.44 ± 0.09
MDA-MB-157	1.22 ± 0.05	0.33 ± 0.07	1.26
MDA-MB-453	1.04 ± 0.16	0.78 ± 0.22	0.75
BT474	0.87 ± 0.11	0.82 ± 0.15	0.88 ± 0.27
MDA-MB-231	0.79 ± 0.06	1.21	0.43
BT483	0.75	1.41	0.55

TABLE 1: Numbers represent real time RT-PCR data correlating to Figures 1 and 2 and represent *bcl-2* message levels. Cells were either treated with hPRL (500 ng/ml) or hPRL-G129R (500 ng/ml). Combination treatment (*PRL+G129R*) is as follows: 200 ng/ml PRL + 1000 ng/ml G129R for 48-h. All values are represented as fold change over the untreated controls and are mean $\pm$ SE. a, n=2-5; b, n=2-4; c, n=2-3.

In summary, a number of apoptosis-related genes that are differentially expressed following treatment with either hPRL or hPRL-G129R have been identified for four different breast cancer cell lines. These data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on *bcl-2* mRNA expression in response to hPRL and hPRL-G129R treatment in eleven cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up regulation of Bcl-2.

Key Research Accomplishments

- Comparison of differential gene expression of four human breast cancer cell lines in response to treatment with human prolactin (hPRL) and its antagonist (hPRL-G129R) using a cDNA microarray representing 204 apoptosis-related genes

- Determination of the importance of the *bcl-2* gene in the response to treatment with (hPRL) or its antagonist (hPRL-G129R) by human breast cancer cell lines

- Quantitative analysis of levels of *bcl-2* mRNA in eleven human breast cancer cells by real time RT-PCR in response to treatment with (hPRL), its antagonist (hPRL-G129R), or a combination of treatments

- Statistical analyses of levels of *bcl-2* mRNA of eleven human breast cancer cells in response to treatment with (hPRL), its antagonist (hPRL-G129R), or a combination of treatments

Reportable Outcomes

Manuscripts:

1. Beck MT, Peirce,SK, and Chen WY. (2002). **Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R.** *Oncogene* (In Press).

2. Chen NY, Holle L, Li W, Peirce SK, Beck MT and Chen WY (2002). ***In vivo* studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R.** *Int. J. Oncology* 20: 813-818.

3. Peirce SK and Chen WY (2001).**Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR.** *J. Endocrinology*, 171, R1-R4

Abstracts and Poster Sessions

Regulation of Bcl-2 Expression by hPRL and Its Antagonist, hPRL-G129R, in Human Breast Cancer Cell Lines.
Susan K. Peirce. Michael T. Beck and Wen Y. Chen
Endo 2002 (The Endocrine Society's 84th Annual Meeting); San Francisco, June 19-22, 2002
Chosen for inclusion in the ENDO 2002 Research Summaries Book (RSB). Each year, the Media Advisory Committee (MED) and the Public Affairs staff of The Endocrine Society identify approximately 125 newsworthy abstracts to include in the RSB.

From An Antagonist Back To An Agonist: Two Wrongs Do Make A Right.
J.F. Langenheim, M.T.Beck, S.K.Peirce, L.Holle and W.Y.Chen
Endo 2002 (The Endocrine Society's 84th Annual Meeting); San Francisco, June 19-22, 2002
Chosen for both oral and poster presentation.

Real Time RT PCR Analysis of Relative Prolactin Receptor (PRLR) Levels in Human Cancer Cell Lines.
S.K. Peirce, R.B. Westberry and Wen Y. Chen.
Endo 2001(The Endocrine Society's 83rd Annual Meeting), Denver, June 20-23, 2001

Conclusions

Our data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on *bcl-2* mRNA expression in response to hPRL and hPRL-G129R treatment in eleven cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up regulation of Bcl-2. It is generally accepted that for cancer therapy one should not design an approach based solely upon increasing death signals, such as chemotherapeutics. Rather, a two-fold approach combining chemotherapeutics with removal of survival factors will result in a more efficient treatment. Our data regarding hPRL-G129R further strengthens its potential therapeutic role in breast cancer therapy.

References

Beck MT, Peirce,SK, and Chen WY. (2002). **Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R.** *Oncogene* (In Press).

Chen NY, Holle L, Li W, Peirce SK, Beck MT and Chen WY (2002). ***In vivo* studies of the anti-tumor effects of a human prolactin antagonist, hPRL-G129R**. *Int. J. Oncology* 20: 813-818.

Peirce SK and Chen WY (2001).**Quantification of prolactin receptor mRNA in multiple human tissues and cancer cell lines by real time RT-PCR.** *J. Endocrinology*, 171, R1-R4

Appendices

See attached abstracts and manuscript.

The Endocrine Society's 84th Annual Meeting

Filename: 852599

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Awards: Travel Grant Awards.
   Do you consider yourself a member of one of the following U.S. underrepresented groups?
   race or ethnic affiliation not entered

Title: REGULATION OF BCL-2 EXPRESSION BY HPRL AND ITS ANTAGONIST, HPRL-G129R, IN HUMAN BREAST CANCER CELL LINES
Susan K Peirce [1]*, Mike T Beck [1] and Wen Y Chen [1][2]. [1]Dept. of Microbiology and Molecular Medicine, Clemson University, Clemson, SC, 29681 and [2]Oncology Research Institute, Greenville Hospital System, Greenville, SC, 29605.

In previous studies, we have shown that the human prolactin antagonist hPRL-G129R is able to induce apoptosis in four human breast cancer cell lines. In the current study, a combination of PCR subtraction and cDNA microarray methodologies was used to examine the gene expression profiles of two estrogen receptor (ER) positive, and two ER negative human breast cancer cell lines following treatment with hPRL or hPRL-G129R. Among the many genes that were found to be differentially expressed, bcl-2 was strongly induced following hPRL treatment in three of the four cell lines tested. To confirm the evidence linking hPRL and bcl-2 expression, a quantitative method of real time RT-PCR and a Bcl-2 ELISA were used to measure bcl-2 mRNA expression levels and Bcl-2 protein levels, respectively. Eleven human breast cancer cell lines were assayed following treatment with hPRL, hPRL-G129R, or a combination of these proteins. We found that hPRL induced, while hPRL-G129R inhibited, bcl-2 mRNA expression in a majority of the cell lines tested, and that the induction of bcl-2 by hPRL was competitively inhibited by hPRL-G129R in most of these cell lines. The pattern of mRNA expression following these treatments correlated to the Bcl-2 protein levels. In particular, MCF-7, T47D and BT549 cells all demonstrated a three to four fold difference in bcl-2 expression levels between hPRL and hPRL-G129R treatments. In contrast, BT474, MDA-MB-231 and BT483 cells showed minimal bcl-2 response to hPRL or hPRL-G129R treatments. There was no correlation between estrogen receptor (ER) status and bcl-2 response to hPRL or hPRL-G129R treatment. The data from these studies suggest that hPRL increases the expression of bcl-2 message and Bcl-2 protein in some human breast cancer cell lines, and hPRL-G129R competitively inhibits bcl-2 expression induced by hPRL in these cell lines.

Keyword 1: Prolactin antagonist
Keyword 2: Oncogene
Keyword 3: Breast cancer

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The Endocrine Society's 84th Annual Meeting

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Title: FROM AN ANTAGONIST BACK TO AN AGONIST: TWO WRONGS DO MAKE A RIGHT

John F Langenheim [1]*, Michael T Beck [1], Susan K Peirce [1], Lori Holle [2] and Wen Y Chen [1][2]. [1]Dept. of Microbiology and Molecular Medicine, Clemson University, Clemson, SC, 29681 and [2]Oncology Research Institute, Greenville Hospital System, Greenville, SC, 29605.

It is generally accepted that the initial step of signal transduction for human growth hormone (hGH) as well as human prolactin (hPRL) is to bind to their respective receptors. The binding process is reported to be sequential: one ligand binds to the first receptor through its binding site one with high affinity and then finds its second receptor through its binding site two with lower affinity resulting in a one ligand/two receptor complex. This ligand induced dimerization of the receptors is essential for hGH and hPRL signal transduction. Amino acid substitution mutation in binding site two of either hGH (hGH-G120R) or hPRL (hPRL-G129R) results in mutants with antagonistic effects both in vitro and in vivo demonstrated by many labs including ours. In our recent attempts to generate a more potent hPRL antagonist with a longer serum half-life, we produced a G129R-G129R homo-dimer using an E. coli expression vector, pET22b. The protein was purified using Q-sepharose anion exchange chromatography and a FPLC system. To our astonishment, the G129R-G129R homo-dimer acts in every aspect as an agonist assayed by STAT5 phosphorylation in human breast cancer cells. We found that the G129R-G129R homo-dimer is able to induce STAT5 phosphorylation in a concentration-dependent manner at a dose range similar to that of wild type hPRL. The induction of STAT5 phosphorylation is not only dose-dependent but also show self-antagonism in high concentration as seen in the case of hPRL. It is interesting to point out that the activation of STAT5 phosphorylation by G129R-G129R homo-dimer can be inhibited by G129R monomer. Our results suggest that as long as there are two binding sites (site 1 plus site 2 in wild type hPRL or site 1 plus another site 1 in G129R-G129R homo-dimer) in one molecule, the ligand serves as an agonist. Our data also suggests that the overall size of the ligand is not a crucial factor (23kd monomer or 46kd dimer) to induce PRL signal transduction. The potential use of homo-dimers of antagonists as longer half-life agonists needs further testing.

Keyword 1: Prolactin antagonist
Keyword 2:
Keyword 3:

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ORIGINAL PAPERS

Regulation of *bcl-2* gene expression in human breast cancer cells by prolactin and its antagonist, hPRL-G129R

Michael T Beck[1], Susan K Peirce[1] and Wen Y Chen*[1,2]

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To gain insight into the molecular basis of human prolactin (hPRL) antagonist induced apoptosis, we compared the differential gene expression profile of four human breast cancer cell lines following treatment with hPRL and its antagonist (hPRL-G129R). Among the genes identified, the *bcl-2* gene was of particular interest. We found that *bcl-2* mRNA was up regulated in three of the four cell lines that were treated with hPRL. To further confirm these results, real time RT–PCR and ELISA analyses were used to detect *bcl-2* mRNA and Bcl-2 protein, respectively, in 11 different breast cancer cell lines after hPRL or hPRL-G129R treatment. Our data suggests that Bcl-2 is up-regulated in response to hPRL stimulation and is competitively inhibited by hPRL-G129R in the majority of the cell lines tested. Thus, we propose that the anti-apoptotic role of hPRL in breast cancer is mediated, at least in part, through regulation of Bcl-2.
Oncogene (2002) 00, 00–00. doi:10.1038/sj.onc.1205637

Introduction

Human PRL is a neuroendocrine polypeptide hormone primarily produced by the lactotrophs in the anterior pituitary gland of vertebrates. It is well established that hPRL is directly involved in the development and differentiation of normal mammary gland in mammalian species (Blackwell and Hammond, 1999; Clevenger *et al.*, 1995; Nagasawa *et al.*, 1985; Topper and Freeman, 1980; Vonderhaar, 1998). Controversy, however, still exists regarding the role of hPRL in human breast cancer. Emerging evidence links hPRL to human breast cancer including: (a) the detection of biologically active hPRL in human breast cancer cells, which suggests that hPRL is produced locally as an autocrine/paracrine growth factor within the mammary gland (Clevenger *et al.*, 1995; Goffin *et al.*, 1996, Goffin

and Kelly, 1997; Vonderhaar, 1999); (b) PRL receptor (PRLR) levels are significantly higher in human breast cancer cells than in normal breast epithelial cells (Kelly *et al.*, 1991); (c) transgenic mice over expressing hPRL have a higher breast cancer incidence (Wennbo *et al.*, 1997) and (d) the hPRL antagonist, hPRL-G129R, slows the growth rate of human breast cancer xymographs in nude mice (Chen *et al.*, 2002). These ① examples support hPRL's role as a mitogen in human breast cancer and suggest that its antagonist may have potential in treating human breast cancer.

Apoptosis plays a critical role in the regulation of cells that are either in a normal or cancerous state of growth. Key regulators that control apoptosis are kept highly controlled by the cells' internal machinery. One of the first and most widely studied regulators of apoptosis to be identified was Bcl-2, which is now known to be a part of a family of related proteins (Adams and Cory, 1998). *bcl-2* is a human proto-oncogene that when overexpressed, will ultimately lead to the inhibition of cell death (Korsmeyer, 1999). It suppresses apoptosis by blocking the release of cytochrome *c*, a major component of cellular respiration, from the mitochondria, thus preventing the activation of caspases, a group of proteases that carry out the process of cell death (Kumar *et al.*, 2000; Yin *et al.*, 1994). In human breast cancer cells, Bcl-2 and Bax, the inhibitor of Bcl-2, are constitutively expressed to tightly regulate apoptosis (Adams and Cory, 1998; Binder *et al.*, 1996; Kumar *et al.*, 2000; Yin *et al.*, 1994). One of many factors leading to breast malignancy is the up-regulation of *bcl-2* gene expression, ultimately resulting in the inhibition of apoptosis (Green and Beere, 1999). There are numerous molecules that can regulate Bcl-2. For example, IL-3 has been shown to increase the expression of *bcl-2* in hematopoietic cell lines (Krumenacker *et al.*, 1998). Studies using Nb2 cells, a rat lymphoma cell line, show that *bcl-2* is up-regulated in immortalized cell lines (Krumenacker *et al.*, 1998; Leff *et al.*, 1996). One of the more relevant studies involving *bcl-2* demonstrated that treatment of Nb2 cells with PRL results in *bcl-2* up-regulation and *bax* down-regulation (Krumenacker *et al.*, 1998). However, there have been no definitive studies linking hPRL to Bcl-2 activity in human breast cancer cells.

The identification of specific genes that are differentially expressed in response to exogenous treatments has been a subject of great interest to many researchers in the past. There are several methods to compare gene

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expression patterns in tissue cells, such as representational difference analysis, differential display, cDNA array hybridization and serial analysis of gene expression (DeRisi *et al.*, 1996; Guiliano *et al.*, 1999; Hakvoort *et al.*, 1994; Oh *et al.*, 1999; Zahn *et al*, 1998). All these methods are able to detect different gene expression profiles, but a newly described technique called suppression subtractive hybridization (SSH) offers additional advantages (Kuang *et al.*, 1998; Yang *et al.*, 2000). Briefly, SSH first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the 'tester' and the reference cDNA is referred to as the 'driver'. Both 'tester' and 'driver' cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The 'tester' cDNAs are then hybridized with 'driver' cDNAs, which have no adaptors on their ends. Suppression PCR, using the adaptors as primers, is then performed to allow exponential amplification of the differentially expressed genes. SSH allows investigators to identify which genes are being turned on or off in one cell type versus another more quickly and easily than other techniques. It is also possible to compare expression profiles of the same cell line by treating a group of cells with a specific compound and using an untreated group as the control. This variation of SSH allows investigators to understand which genes are being expressed in response to a specific treatment of choice. SSH is valuable because it includes an amplification step and selection step that other methods do not, thus increasing the levels of differentially expressed genes while decreasing the levels of housekeeping genes that result in unnecessary background. The introduction of the cDNA microarray makes it possible to identify genes in a much more efficient manner. This emerging technique has proven to be an essential tool when attempting to identify which genes are responding to a certain condition (Oh *et al.*, 1999; Yang *et al.*, 2000). By combining these two methods it is possible to obtain and identify differentially expressed genes with precision (Beck *et al.*, 2001).

In this study, we examined the profile of apoptosis related genes expressed by four human breast cancer cell lines upon treatment with either hPRL or hPRL-G129R. It was found that *bcl-2* gene expression was increased following treatment of breast cancer cells with hPRL in both estrogen receptor (ER) positive cell lines and one of two ER negative cell lines tested. To confirm the evidence linking hPRL and Bcl-2, a quantitative method of RT–PCR and a Bcl-2 ELISA were used to measure both *bcl-2* mRNA expression levels and protein levels in 11 human breast cancer cell lines after treatment with hPRL or hPRL-G129R. The data from these studies suggests that hPRL acts as an apoptosis inhibitor by increasing the expression of Bcl-2 in human breast cancer and that hPRL-G129R competitively inhibits Bcl-2 induction by hPRL.

Results

Profile of apoptosis genes in response to hPRL or hPRL-G129R in four human breast cancer cell lines

Comparisons of the relevant apoptosis related genes expressed in human breast cancer cells are shown in Figure 1. hPRL-G129R treated T-47D and MCF-7 cells shown in Figure 1a,c and hPRL treated in Figure 1b,d, respectively. It appears that in both cases the only gene that was up regulated is *bcl-2* (10-F). In Figure 1a, T-47D cells treated with hPRL-G129R exhibited a strong up-regulation of caspases, such as caspases-3 (11-A), -4 (11-F), -7 (11-I), -9 (11-L) and -10 (11-M). The Bcl-2 binding protein, BNIP$_3$, was expressed in all but the MDA-MB-468 after hPRL-G129R treatment (Table 1). In the T-47D cells, the genes related to death receptors such as serine-threonine kinase 1 (12-H), DAXX (12-I), tumor necrosis factor-related apoptosis inducing ligand (14-F) and death domain receptor 3 (15-G) were up-regulated after hPRL-G129R treatment, although the gene for caspase-8 (11-J and 11-K), which is normally associated with death receptors, has not. In MCF-7 cells the gene *BAD* (10-O in Figure 1c), an important member of the Bcl-2 family of proteins, was differentially expressed after hPRL-G129R treatment. There was no evidence of caspase expression in MCF-7 cells after hPRL-G129R treatment.

Table 1 summarizes all apoptosis related differentially expressed genes in T-47D, MCF-7, BT-549 and MDA-MB-468 cells treated with either hPRL or hPRL-G129R that were probed on an apoptosis microarray. To our knowledge, this is the first time that a list of apoptosis related differentially expressed genes has been compiled for these four breast cancer cells after treatment with hPRL and its antagonist.

Quantitative RT–PCR measurement of bcl-2 mRNA in 11 human breast cancer cell lines

To confirm that hPRL induced the expression of *bcl-2*, quantitative real time RT–PCR was used. Figure 2a represents direct real time RT–PCR output from T-47D cells treated with either hPRL or hPRL-G129R and compared to the untreated control. *bcl-2* message levels were clearly elevated in the hPRL treated samples as indicated by the amplification curves shift to the left and decrease in the hPRL-G129R treated samples as indicated by the amplification curves shift to the right, relative to untreated samples. All samples were normalized to equivalent levels of β-actin mRNA (Figure 2b). Table 2 represents the *bcl-2* levels from multiple quantitative real time RT–PCR runs relative to normalized levels of β-actin. The data is presented as levels of *bcl-2* in all 11 cell lines treated with hPRL, hPRL-G129R or a combination of hPRL and hPRL-G129R, and were compared to levels of *bcl-2* in the untreated controls. The responses are graphed as the per cent change of the experimental response to the untreated control $\pm$ s.e. and shown in Figure 3. In MCF-7 and MDA-MB-134 cells, hPRL treatment
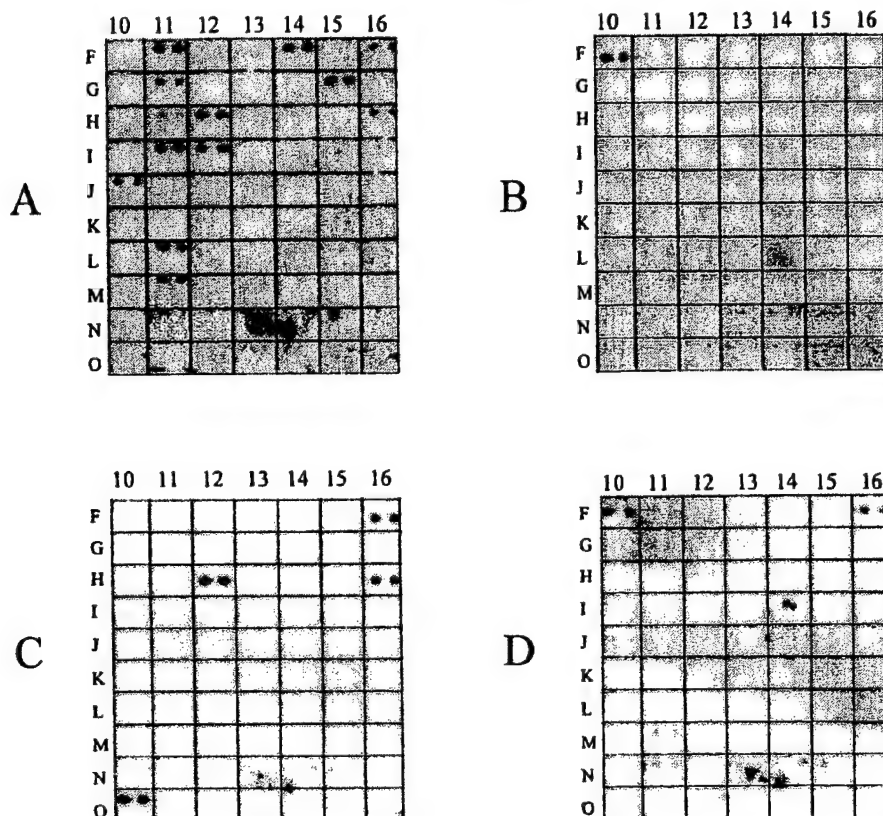
Figure 1 Representation of SSH and microarrays. Three micrograms of DIG labeled differently expressed cDNAs were probed onto each microarray and detected by means of chemiluminescence. T-47D cells treated with hPRL-G129R (a) and hPRL (b) and MCF-7 cells treated with hPRL-G129R (c) and hPRL (d) are shown as a representative of the microarrays

resulted in a highly significant ($P<0.01$) up-regulation of *bcl-2* message, while in BT-549 and T-47D cells the per cent change was significant ($P<0.05$). In the remaining seven cell lines, *bcl-2* message levels were not significantly different from the untreated controls. Treatment with the antagonist resulted in significantly ($P<0.05$) decreased expression of *bcl-2* message in four of the cell lines (MCF-7, T-47D, BT-549 and MDA-MB-157) with no significant change in the other cell lines. A modest increase in *bcl-2* message expression was observed in four cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-231, BT-483) following hPRL-G129R treatment. In seven cell lines a combination treatment of hPRL-G129R and hPRL resulted in lower levels of *bcl-2* expression than the hPRL treatment alone (MCF-7, T-47D, MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483). The combination treatment significantly ($P<0.05$) reduced *bcl-2* expression levels in MCF-7 and T-47D cell lines, whereas MDA-MB-134, MDA-MB-453, BT-474, MDA-MB-231 and BT-483 cells show an insignificant decrease in the level of Bcl-2.

Overexpression of Bcl-2 protein in various cell lines

To further confirm that Bcl-2 was upregulated in human breast cancer cells, a Bcl-2 ELISA was performed on all 11 cell lines. Data is presented as per cent change of Bcl-2 levels in cells treated with hPRL (100 ng/ml) or in cells treated with hPRL-G129R (500 ng/ml) and the combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml) over cells with no treatment. Figure 4a illustrates that six of the 11 human breast cancer cell lines tested showed a highly significant increase ($P<0.01$) in Bcl-2 protein levels after treatment with hPRL. T-47D, MDA-MB-157 and MDA-MB-134 demonstrated the highest levels of Bcl-2 with an increase of approximately 175% over untreated cells. MCF-7, BT-549 and MDA-MB-483 all exhibited levels of Bcl-2 with an increase of approximately 125% over the untreated cells. MDA-MB-468, MDA-MB-453 and BT-474 demonstrated a less significant ($P<0.05$) per cent change of Bcl-2 levels after treatment with hPRL. The remaining two cell lines, MDA-MB-436 and MDA-MB-231, did not demonstrate a significant level of Bcl-2 increase after treatment with hPRL. It is clear from Figure 4b that upon, treatment with hPRL-G129R (500 ng/ml), there was a highly significant ($P<0.01$) decrease in the levels of Bcl-2 in all 11 cell lines. The combination treatment of hPRL and hPRL-G129R demonstrated a highly significant ($P<0.01$) decrease of Bcl-2 protein in nine of the 11 cell lines (Figure 4c). MDA-MB-436 and BT-

Table 1 Differentially expressed gene profile for four human breast cancer cell lines

| | Cell lines and their treatments | | | | | | | |
| | (PRLR+ and ER+) | | | | (PRLR+ and ER−) | | | |
| | T-47D | | MCF-7 | | BT549 | | MDA-MB-468 | |
Genes	hPRL	G129R	hPRL	G129R	hPRL	G129R	hPRL	G129R
Cell cycle-regulating proteins and kinases								
Cell division cycle (CDC)-like kinase 1						+		
Serine/threonine protein kinase 1	+		+					
Cyclin dependent kinase (CDK)-G2	+						+	
CDK 4 inhibitor 2D						+		
CDC10 protein homolog	+			+	+		+	
Ubiquitin-conjugating enzyme E2						+		
CDC16HS		+						
MAP kinase 3				+			+	
MAP kinase 1					+			
MAPKK 1		+				+		
MAPKK 5							+	
MAPKK 10							+	
Peptidyl-prolyl cis-transisomerase nima-interacting 1					+			
Retinoblastoma-binding protein 4					+		+	
E2F dimerization partner 1								
Bcl-2 family proteins and caspases		+						
B-cell lymphoma protein 2 (Bcl-2)	+		+		+			
Bcl-2-associated death promoter (BAD)				+		+		+
Bcl-2 binding protein (BNIP₃)		+		+		+		
BAK1								+
BID3		+						
Caspase-3		+				+		
Caspase-4		+						
Caspase-7		+				+		+
Caspase-8								+
Caspase-9		+						
Caspase-10		+						
Death receptors/ligands and apoptosis associated proteins								
TNF receptor 1 associated death domain protein				+		+		
TNF receptor-associated factor 6						+		
Death receptors/ligands and apoptosis associated proteins								
Receptor interacting protein (RIP)		+		+		+		+
DAXX		+						+
TNF-alpha converting enzyme		+						
TNF-related apoptosis inducing ligand		+						
Caspase Death Domain								+
Death domain receptor 3		+						
Insulin-like growth factor-binding protein 2 (IGFBP-2)	+			+				
IGFBP-4				+				
Fas-activated serine/threonine kinase (FAST)		+		+				
Nuclear kappa factor-B DNA binding subunit			+					
Glutathione peroxidase 1						+		
Glutathione S-transferase theta 1						+		

Cells were either treated with hPRL or hPRL-G129R as indicated. Genes that were differentially expressed are represented with a '+' symbol below the treatment that stimulated their expression and when not expressed the field was left blank as shown

474 cells show a less significant ($P<0.05$) per cent change of Bcl-2 levels after the combination treatment.

Discussion

Apoptosis, or programmed cell death, is a means of regulating cellular growth and differentiation without the inflammatory response generally induced by necrotic cell death (Adams and Cory, 1998). During mammary gland development, and more importantly involution, key apoptosis-inducing Bcl-2 family proteins, such as Bax, Bad and Bcl-w are up regulated (Li, 1997; Schorr et al., 1999a), and Bcl-2 appears to act as a regulator of Bax levels. It has been well established that decreased levels of Bax are correlated to increased levels of Bcl-2 and that this Bax/Bcl-2 ratio is also critical to normal breast development (Reed, 1998; Green, 2000; Adams and Cory, 1998). Increases in levels of Bcl-2 appear to be more important to cell survival than the down-regulation of Bax (Schorr et al., 1999b). The bcl-2 oncogene has been shown to have an anti-apoptotic function and may play a role in tumorigenesis by raising the threshold for apoptosis (Adams and Cory, 1998). In our previous studies, we reported that an hPRL
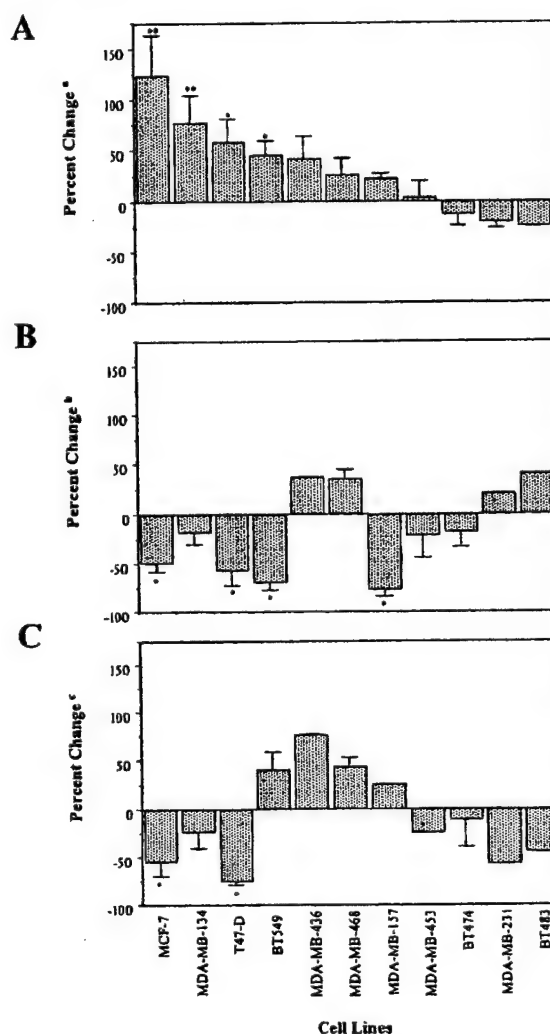
A

B
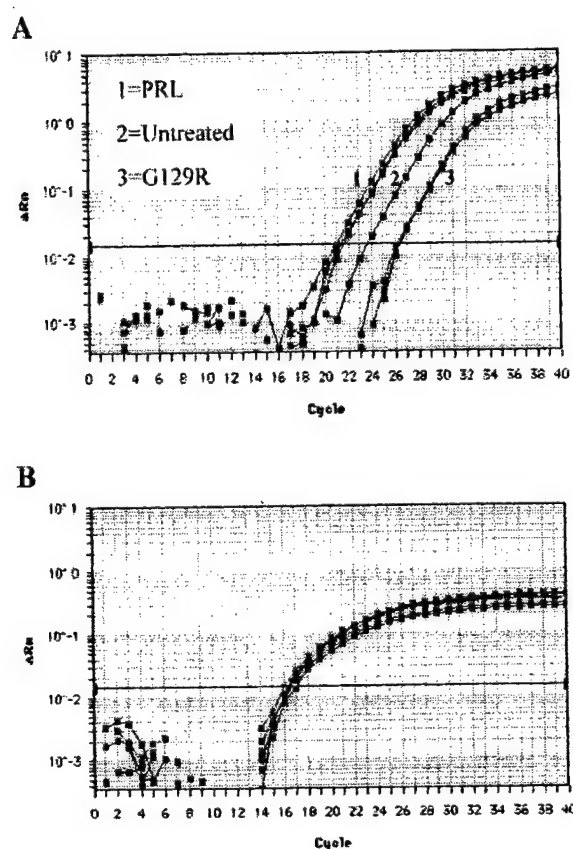
Figure 2 Real time RT–PCR spectral output measuring *bcl-2* levels in T-47D cells treated with 500 ng/ml hPRL (left-hand curve) or 500 ng/ml of hPRL-G129R (right-hand curve), relative to the untreated control (central curve) (a). (b) Represents the Real time RT–PCR spectral output measuring β-actin levels in untreated, hPRL and hPRL-G129R treated T-47D cells

Figure 3 Real-time quantitative measurement of *bcl-2* mRNA levels in 11 breast cancer cell lines in response to 48 h treatments with hPRL (500 ng/ml; a), hPRL-G129R (500 ng/ml; b) and a combination treatment of hPRL (200 ng/ml) and hPRL-G129R (1000 ng/ml; c). Levels are represented as the fold change over the untreated controls, and numbers are presented as mean $\pm$ s.e. **a**, $n=2-5$; **b**, $n=2-4$; **c**, $n=2-3$. **$P<0.01$ versus basal levels of Bcl-2; *$P<0.05$ versus basal levels of Bcl-2

Table 2 Fold difference of *bcl-2* message of treatments over untreated cells

| Cell line	PRL[a]	G129R[b]	PRL+G129R[c]
MCF-7	2.25 ± 0.19	0.50 ± 0.08	0.45 ± 0.15
MDA-MB-134	1.78 ± 0.27	0.82 ± 0.12	0.76 ± 0.17
T47-D	1.59 ± 0.29	0.43 ± 0.15	0.24 ± 0.03
BT549	1.46 ± 0.14	0.30 ± 0.07	1.41 ± 0.38
MDA-MB-436	1.42 ± 0.28	1.38	2.77
MDA-MB-468	1.27 ± 0.15	1.26 ± 0.09	1.44 ± 0.09
MDA-MB-157	1.22 ± 0.05	0.33 ± 0.07	1.26
MDA-MB-453	1.04 ± 0.16	0.78 ± 0.22	0.75
BT474	0.87 ± 0.11	0.82 ± 0.15	0.88 ± 0.27
MDA-MB-231	0.79 ± 0.06	1.21	0.43
BT483	0.75	1.41	0.55

Numbers represent real time RT–PCR data correlating to Figure 3 and represent *bcl-2* message levels. Cells were either treated with hPRL (500 ng/ml) or hPRL-G129R (500 ng/ml). Combination treatement (*PRL+G129R*) is as follows: 200 ng/ml PRL + 1000 ng/ml G129R for 48 h. All values are represented as fold change over the untreated controls and are mean ± s.e. [a]$n=2-5$; [b]$n=2-4$; [c]$n=2-3$

of apoptosis (Chen *et al.*, 1999), suggesting that the role of hPRL in breast cancer cells may be anti-apoptotic. We have also shown that hPRL down-regulates TGFβ1 (apoptotic factor) and up-regulates TGFα (survival factor) secretion in a dose-dependent manner in human breast cancer cells (Ramamoorthy *et al.*, 2001). More importantly, hPRL-G129R up-regulates TGFβ1 and down-regulates TGFα. In the same study it was also shown that caspase-3 is up regulated by hPRL-G129R. In the present study, we looked at a vast array of genes within breast cancer cells that are responding to treatment with hPRL and hPRL-G129R. We provide evidence that the potential tumorigenic effects (autocrine and paracrine) of hPRL may be mediated through the up-regulation of Bcl-2.

antagonist, hPRL-G129R, is able to inhibit human breast cancer cell proliferation through the induction

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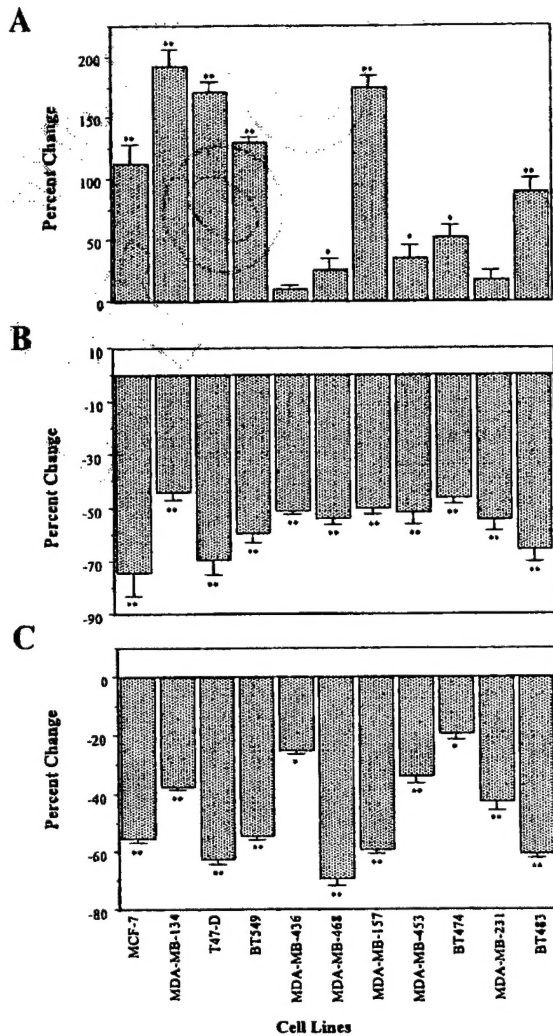
Figure 4 Expression of the Bcl-2 protein in various cell lines. Cells were treated with either hPRL (100 ng/ml; a), hPRL-G129R (500 ng/ml; b) or a combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml, c) for 48 h, lysed and ELISAs were performed to determine the relative levels of Bcl-2 present in the cells. All cells were assayed in triplicate and levels of Bcl-2 was determined using a standard curve prepared using known Bcl-2 standards from the manufacture. Fold induction and inhibition were determined using untreated cells as a control for hPRL treated cells and hPRL treated cells as a control for the combination treatment. Cell lines are arranged from left to right in order of increasing bcl-2 mRNA message level (Figure 3.). Numbers are presented mean $\pm$ s.e. of at least three independent experiments. $**P<0.01$ versus basal levels of Bcl-2; $*P<0.05$ versus basal levels of Bcl-2

The microarray studies using four breast cancer cell lines implied the heterogeneous nature of human breast cancer (Table 1). It is apparent in the gene expression patterns of four cell lines that the gene bcl-2 is overexpressed in T-47D, MCF-7 and BT-549 breast cancer cells following hPRL treatment. In response to hPRL-G129R treatment, BNIP3, a 19-KD dimeric mitochondrial protein that binds to Bcl-2 and suppresses its anti-apoptotic activity (Chen et al., 1997), is up-regulated in three of the four cell lines (Table 1). These data suggest an additional role for hPRL in regulation of Bcl-2 activity in breast cancer cells. To further confirm this finding, we utilized 11 breast cancer cell lines and treated them with hPRL, hPRL-G129R or a combination of the two and measured bcl-2 mRNA as well as Bcl-2 protein levels. Increases in bcl-2 mRNA levels in four cell lines (MCF-7, MDA-MB-134, T-47D and BT-549) are highly significant $(P<0.01)$ following treatment by hPRL and are down regulated by hPRL-G129R treatment in five cell lines (MCF-7, MDA-MB-134, T-47D and BT-549 and MDA-MB-157) (Figure 3). However, the response of Bcl-2 to the treatment of hPRL or hPRL-G129R was most apparent at the Bcl-2 protein level (Figure 4). Eight of the 11 cell lines demonstrated a highly significant increase of Bcl-2 protein after hPRL treatment $(P<0.01)$. The maximum increase in Bcl-2 protein after a single dose treatment with hPRL was several fold higher than the basal level in untreated controls. In contrast, the decrease of Bcl-2 levels in response to treatment by hPRL-G129R was highly significant $(P<0.01)$ in all 11 cell lines (Figure 4). The cell lines (MDA-MB-436, MDA-MB-468, MDA-MB-453, BT-474, MDA-MB-231 and BT-483) demonstrated no statistically significant bcl-2 response to hPRL treatment at the mRNA level, and showed a relatively lower response at the protein level. This would suggest that in these cell lines hPRL plays a less significant role in maintaining proliferation via the Bcl-2 protein. The discrepancy between the Bcl-2 mRNA levels and the protein levels in some cell lines after treatment may be attributed to many factors, which include the general instability of mRNA in the cells, relative to protein, where Bcl-2's half-life is greater than 10 h (Merino et al., 1994).

The cell lines used in the cDNA subtraction experiments have been reported to produce PRL as an autocrine/paracrine growth factor, from the work of others (Vonderhaar et al, 1995; Shaw-Bruha et al., 1997; Yamauchi et al., 2000). We do not have evidence, at this point, that hPRL-G129R contains an intrinsic ability to elicit novel signal transduction of its own. Rather, we believe that the down regulation of the bcl-2 expression by hPRL-G129R is through the competitive inhibition of the effects induced by endogenous PRL. These findings are consistent with those of Llovera et al. (2000), who have found that the hPRL-G129R does not activate any specific signaling molecules in their systems.

We found no obvious correlation between the published (Ormandy et al., 1997) ER status and the bcl-2 response following hPRL and hPRL-G129R treatment. For example, both the ER negative BT-549 and the most ER positive MDA-MB-134 cell lines demonstrated high levels of bcl-2 response, while the strongly ER positive cell line BT-483 and the ER negative MDA-MB-231 cell lines had the lowest responses. In our previous work, we determined relative PRLR mRNA expression levels in 11 breast

cancer cell lines (Peirce and Chen, 2001). In this study, the levels of *bcl-2* expression do not appear to have a linear correlation with that of PRLR mRNA. This might be attributed to the fact that there are multiple intracellular signaling mechanisms involved in regulation of *bcl-2* expression. For example, there is recent evidence of cross-talk between PRL receptor and HER2/neu through phosphorylation of Jak2 that leads to the activation of MAP kinases in breast cancer cell lines (Yamauchi *et al.*, 2000). Related to this finding, we have seen that MAP kinases are, in fact, up regulated by PRL treatment in three out of four cell lines (Table 1). Further work in this area should help to identify the specific pathways by which cellular apoptosis is regulated by hPRL and hPRL-G129R.

cDNA microarrays provide a powerful means for identifying genes differentially expressed in cells after certain alterations. However, the vast amount of information revealed after array analyses often leaves more questions than answers. In this study, we decided to focus on one gene, *bcl-2*, after analysing initial results of the cDNA subtraction and array analysis. There are clearly many questions to be addressed (Table 1). For example, we found that there was no evidence of caspase expression in MCF-7 cells following treatment by hPRL-G129R, whereas caspase expression is up regulated in T-47D, BT-549 and MDA-MB-468 cells. We previously reported that caspase-3 activity was up regulated in T-47D cells following treatment with hPRL-G129R (Ramamoorthy *et al.*, 2001). There is a lack of caspase mRNA expression and the appearance of a more direct link to Bcl-2 related apoptosis via the BAD (Bcl-2 associated death promoter) protein in MCF-7 cells (Figure 1c). During apoptosis, BAD has been shown to bind to Bcl-2 and release it from the mitochondrial membrane resulting in total cellular disruption. BAD operates upstream of the caspase pathway suggesting that MCF-7 cells activate apoptosis via a different pathway utilized by the other three breast cancer cell lines. It has been previously shown that MCF-7 cells lack caspase-3 entirely due to a 47-base pair deletion within the CASP-3 gene, although this cell line is still able to undergo apoptosis even in the absence of DNA fragmentation (Janicke *et al.*, 1998; Liang *et al.*, 2001). Thus, our data tentatively identifies one component of the caspase-3 independent signaling pathway that MCF-7 cells may use to trigger apoptosis. It is also interesting to point out that a death domain protein, the receptor-interacting protein (RIP), is differentially expressed in all four cell lines following hPRL-G129R treatment. It has been reported that over-expression of RIP induces both NF-κB activation and apoptosis (Hsu *et al.*, 1996). Our results may be of interest when investigating the death domain proteins and death domain receptors in relation to hPRL and hPRL-G129R.

In summary, a list of apoptosis related genes that are differentially expressed following treatment with either hPRL or hPRL-G129R has been compiled for four different breast cancer cell lines. These data will allow for future studies of specific genes that are involved with cellular proliferation or apoptosis in human breast cancer. By focusing on Bcl-2 mRNA or protein expression in response to hPRL and hPRL-G129R treatment in 11 cell lines, we provide further evidence that the anti-apoptotic effects of hPRL in breast cancer are likely mediated through the up regulation of Bcl-2. It is generally accepted that, for cancer therapy, one should not design an approach based solely upon increasing death signals, such as chemotherapeutics. Rather, a twofold approach combining chemotherapeutics with removal of survival factors will result in a more efficient treatment. Our data regarding hPRL-G129R further strengthens its potential therapeutic role in breast cancer therapy.

Materials and methods

Cell lines and growth conditions

Five ER positive (T-47D, MDA-MB-134, BT-474, MDA-MB-483, MCF-7) and six ER negative human breast cancer cell lines (BT-549, MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). T-47D, BT-549, BT-474 and MDA-MB-483 cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) and 100 μg/ml gentamycin (for all media used) (Hyclone). BT-459 cells were supplemented with 200 IU of Insulin (Sigma, St. Louis, MO, USA). MDA-MB-483 cells were supplemented with 0.2 mM sodium pyruvate, 2 mM HEPES buffer and 200 IU Insulin (Sigma). MCF-7 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS. MDA-MB-231, MDA-MB-134, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157 cells were maintained in Leibovitz L-15 (Life Technologies) media. MDA-MB-231, MDA-MB-436 and MDA-MB-453 cells were supplemented with 10% FBS with the addition of 200 IU of Insulin for MDA-MB-436. MDA-MB-468 and MDA-MB-157 cells were grown in the presence of 15% FBS and MDA-MB-134 in the presence of 20% FBS. Cell lines T-47D, BT-549, BT-474, MDA-MB-483, MCF-7 and MDA-MB-231 were grown at 37°C in a humid atmosphere in the presence of 5% CO_2. MDA-MB-134, MDA-MB-453, MDA-MB-468, MDA-MB-436 and MDA-MB-157 cells were grown at 37°C in a humid atmosphere in the absence of CO_2.

PCR-Select cDNA suppression subtraction hybridization

Before experiments, cells were split into three groups of 10 T75 flasks and grown in their specific medium supplemented with 10% charcoal-stripped fetal bovine serum (CSS) until 80% confluent. Approximately 1×10^8 cells from each group were treated with either 500 ng/ml of hPRL (hPRL was kindly supplied by Dr AF Parlow, National Hormone and Pituitary Program, NIH, USA) or 500 ng/ml of hPRL-G129R in cell specific media supplemented with 1% CSS. The untreated control cells were cultured in their respected medium supplemented with 1% CSS. All cells were treated for 48 h and immediately harvested for mRNA extraction. Polyadenylated mRNA was isolated using the Micro-Fast Track 2.0 kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA yield was determined by

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measuring absorbency at 260 nm. SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) as previously described (Beck et al., 2001). Three micrograms of purified cDNAs from the subtraction hybridizations were random primed labeled with alkali labile digoxigenin-dUTP using the DIG DNA Labeling Kit (Roche Molecular Biochemical's, Mannheim, Germany) according to the manufacturer's protocol.

cDNA microarrays

The Atlas™ human apoptosis array (Clontech) was used for all microarray analyses. The array is a nylon membrane that contains all known apoptosis related genes (205 cDNAs) spotted onto the surface in duplicate. Membranes were pre-hybridized with DIG Easy Hyb solution (Roche) overnight at 37°C in a hybridization incubator with gentle rotation. DIG-labeled probes were purified and resuspended appropriately in TE (pH 8.0). The probes were boiled for 10 min and placed on ice for 5 min. After prehybridization, the DIG-labeled probe was added to the microarray membrane in a total volume of 5 ml of fresh DIG Easy Hub and allowed to hybridize overnight at 68°C in a hybridization incubator with gentle rotation. Membranes were washed twice at 38°C for 5 min in $2 \times$ SSC, 1% SDS, and twice at 68°C for 15 min in $0.1 \times$ SSC, 0.5% SDS. DIG-labeled cDNAs on the hybridized Atlas™ membranes were detected by chemiluminescence using the DIG luminescent detection kit (Roche) according to the manufacturer's specifications using CSPD® as the chemiluminescent substrate. After incubation with CSPD®, membranes were placed in an autoradiography cassette and incubated at 37°C for 15 min to enhance the exposure and then exposed to Kodak Biomax™-MR film at room temperature (all hybridizations were carried out in duplicate). It was determined that using 3 μg of differentially expressed cDNAs for labeling and probing the microarrays was the correct amount for less background and optimal brightness for gene identification. Each array was exposed to the film for various periods of time to allow for the correct exposure to be captured.

Real time quantitative RT–PCR

Before treatment, 11 breast cancer cell lines were depleted of serum for 3 to 4 days in their respective medium supplemented with 1% CSS. Approximately $0.5-1 \times 7^7$ cells from each group were treated with 500 ng/ml of hPRL, 500 ng/ml of hPRL-G129R or a 1000 ng:250 ng ratio of hPRL-G129R to hPRL in cell specific medium supplemented with 1% CSS. The untreated control cells were cultured in cell specific medium supplemented with 1% CSS. All cells were treated for 48 h and harvested for total RNA extraction using the RNAqueous (Ambion, Austin, TX, USA) RNA isolation kit.

A one-step real time reverse transcription PCR (RT–PCR) technique was used to determine relative expression levels of bcl-2 mRNA using the ABI Perkin Elmer Prism 7700 Sequence Detection System (Perkin-Elmer Biosystems, Foster City, CA, USA). The reaction mix included a pre-developed TaqMan® assay mixture containing both forward and

reverse bcl-2 specific primers and a 100 nm final concentration of the bcl-2-specific probe labeled with FAM reporter fluorescent dye (Perkin-Elmer 4319432F). A one-step reaction mixture provided in the TaqMan® Gold RT–PCR Kit (Perkin-Elmer) was used for all amplifications (5.5 mM $MgCl_2$, 50 mM KCl, 0.01 mM EDTA, 10 mM Tris-HCl pH 8.3, 300 μM deoxyATP, 300 μM deoxyCTP, 300 μM deoxyGTP, 600 μM deoxyUTP, 0.025 U/ml AmpliTaq Gold DNA polymerase, 0.25 U/ml MultiScribe Reverse Transcriptase, 0.4 U/ml RNase inhibitor). Cycle parameters for the one-step RT–PCR included a reverse transcription step at 48°C for 30 min followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. Four hundred to 1500 ng of total RNA was used per reaction. The housekeeping gene β-actin was used for internal normalization. Each reaction was carried out in triplicate for each PCR run, and each run was repeated two to five times. Data are expressed as the mean $\pm$ s.e.

Bcl-2 ELISA

A Bcl-2 ELISA (Oncogene Research Products, Boston, MA, USA) analysis was performed according to manufacturer's instructions. Briefly, all 11 cell lines were treated with hPRL (100 ng/ml), hPRL-G129R (500 ng/ml) or combination of hPRL (100 ng/ml) and hPRL-G129R (500 ng/ml) for 48 h in depleted media specific for each cell line described previously. Controls for all treatments were those of untreated cells in their specific defined media. For each cell line, approximately 5×10^6 cells were resuspended in 1 ml of resuspension solution (50 mM Tris, 5 mM EDTA, 0.2 mM PMSF, 1 μg/ml pepstatin and 0.5 μg/ml leupeptin; pH adjusted to 7.4). Two hundred microliters of antigen extraction agent (Oncogene) were added to the cell suspensions and incubated on ice for 30 min. Cell debris was centrifuged and supernatant was frozen until use. Each supernatant was diluted 1:1 to obtain an optimal reading in the range of the standards. Standards were performed in duplicate. Analyses were repeated three times and data are expressed as the mean $\pm$ s.e.

Statistical analyses

All values are given as mean $\pm$ s.e. Statistical analysis was performed using the program StatsDirect version 1.9.8 (CamCode, Cambridge, UK) with one way ANOVA and a Tukey-Multiple Comparison test. P-values less than 0.01 were considered highly statistically significant and P values that were less than 0.05 were considered statistically significant.

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